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A Simple Spectrophotometric Assay of Carboxypeptidase N (Kininase I) in Human Serum

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Summary: Kininase I (carboxypeptidase N; EC 3.4.17.3) consists of carboxypeptidase N_1 (CN_1) and carboxypeptidase N_2 (CN_2); these two enzymes can be differentiated by their activities towards hippuryl-*L*-arginine and hippuryl-*L*-lysine, respectively. A spectrophotometric assay for both carboxypeptidases in human serum is described and the biochemical behaviour of these enzymes investigated. The pH optima are found to be 8.4 for CN_1 and CN_2. The *Michaelis-Menten* constants are: CN_1 4.59 ± 0.03 mmol/l; CN_2 37.26 ± 3.49 mmol/l. CN_2 can be inhibited by EDTA (76%), dimercaprolum (97%) and phenanthroline (98%). Diisopropylfluorophosphate has no influence on both enzymes. Elevated haemoglobin only interferes with CN_1 measurements, and high bilirubin concentrations slightly alter the activity of both enzymes. High CN_1 activities were found in sera of patients with sarcoidosis, and elevated CN_2 activities were found in lung cancer.

Eine einfache spektralphotometrische Methode zur Bestimmung der Carboxypeptidase N (Kininase I) im menschlichen Serum

Zusammenfassung: Die Carboxypeptidase N (CN; EC 3.4.17.3), auch Kininase I genannt, läßt sich aufgrund ihres unterschiedlichen biologischen Verhaltens in CN_1 mit Hippuryl-*L*-arginin und CN_2 mit Hippuryl-*L*-lysin als Enzymsubstrat unterscheiden. Beide im Serum enthaltenen Enzyme spalten die terminalen Aminosäuren ab und lassen dadurch Hippursäure entstehen, deren Konzentration spektralphotometrisch gemessen wird. CN_1 und CN_2 wurden biochemisch charakterisiert. Das pH Optimum lag bei 8,4. Die *Michaelis-Menten*-Konstante betrug: CN_1 4,59 ± 0,03 mmol/l, CN_2 37,29 ± 3,49 mmol/l. CN_2 wurde zu 76% durch EDTA, Dimercaprol (97%) und Phenanthrolin (98%) inhibiert. Diisopropylfluorophosphat hatte keinen Einfluß auf beide Enzyme. Erhöhtes Hämoglobin interferierte bei der CN_1, Bilirubin zeigte nur eine geringe Interferenz. Klinische Bedeutung scheint der CN_1 bei der Lungensarkoidose zuzukommen, bei der die höchsten Aktivitäten im Gegensatz zu anderen Lungenerkrankungen gefunden wurden. Beim Lungenkarzinom war CN_2 erhöht.

Introduction

An enzyme capable of rapidly releasing carboxyl-terminal lysine and arginine from peptides has been found in the bovine pancreas (1). This enzyme was named carboxypeptidase B (EC 3.4.17.3) or basic carboxypeptidase and was also purified (2) and characterized (3, 4). Other tissues also have carboxypeptidase B activity (5, 6). In human plasma, carboxypeptidase B-like activity was found, which differs from the isolated pancreas enzyme in molecular weight, the velocity of hydrolysis of lysine-containing substrates compared to arginine-containing substrates, and inhibition patterns (7). Additionally, it inactivates the nonapeptide bradykinin by removing the C-terminal arginine; therefore it is also called kininase I. In human plasma, carboxypeptidase N has a molecular weight of 280000. Left standing it dissociates to subunits of 45000 and 90000 (8). The plasma enzyme activity was low in some diseases of

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the liver, which indicate that the liver and not the pancreas is the site of enzyme synthesis. The physiological and pathophysiological role of this aminopeptidase in human body fluid has not yet been thoroughly clarified. One reason for the lack of interest in this enzyme may be that the known methods are not routinely practiced in clinical laboratories. Therefore we developed a simple spectrophotometric method for the determination of this carboxypeptidase. This assay is based on the estimation of hippuric acid formed by carboxypeptidase N from two synthetic substrates.

Carboxypeptidase N_1 (CN_1) cleaves the synthetic substrate hippuryl-*L*-arginine, while carboxypeptidase N_2 (CN_2) is used for splitting the hippuryl-*L*-lysine. After biochemical characterisation of both enzymes, this method was applied to a large number of untreated sera in various lung diseases.

Materials and Methods

Reagents

Hippuric acid, hippuryl-*L*-arginine, hippuryl-*L*-lysine, diisopropylfluorophosphate, ethylenediaminetetra-acetic acid (EDTA), dimercaprolum, phenanthroline, bilirubin and haemoglobin were purchased from Sigma, Munich.

All other chemicals of high purity were obtained from Merck, Darmstadt. Test tubes were from Sarstedt, Stuttgart. In all assays distilled and deionized water was used.

0.05 mol/l hippuryl-*L*-arginine solution, molecular weight 335.4, was prepared by dissolving 167.7 mg hippuryl-*L*-arginine in 10 ml of 0.5 mol/l potassium phosphate buffer, pH 8.4; 0.1 mol/l hippuryl-*L*-lysine solution, molecular weight 307.3, was mixed by dissolving 307.3 mg hippuryl-*L*-lysine also in 10 ml of the same buffer. The buffer was prepared by mixing solution A (containing 87 g/l (0.5 mol/l) K_2HPO_4 and 43.8 g/l (0.75 mol/l) NaCl) and solution B (containing 68.0 g/l (0.5 mol/l) KH_2PO_4 and 43.8 g/l (0.75 mol/l) NaCl). A sufficient quantity of solution B was added to 100 ml of solution A to achieve a pH of 8.4 at 20 °C.

The buffer was stored at 4 °C, which caused some precipitation, but the precipitate redissolved at 20 °C.

Serum samples

Blood was collected from healthy controls and from patients with various diseases under the following conditions: Arm venipuncture was performed at 7.30 a.m. after fasting for 2 h and bed rest, with no administration of drugs during the preceding 24 h. The healthy donors had 1/2 h bed rest. The blood was allowed to clot in a 10 ml polypropylene test tube. Blood was centrifugated for 10 min at 1200 *g* in a Heraeus cryofuge at 4 °C, and the supernatant was carefully removed and stored at −20 °C until assay.

Incubation procedure

50 µl serum for measurement of CN_1, or 25 µl serum for CN_2, was added to 200 µl of substrate buffer solution containing hippuryl-*L*-arginine or hippuryl-*L*-lysine in duplicated 4 ml polypropylene test tubes, tightly covered, vortexed for 15 s and incubated for 60 min at 37 °C in a temperature controlled shaker water bath. The enzymatic reaction was stopped by addition of 250 µl of 1 mol/l HCl solution and vortexed for 15 s. The blanks were similary treated except that after addition of serum the enzymatic reaction was stopped at once by 250 µl of 1 mol/l HCl before incubation.

All samples were removed from the water bath and placed in crushed ice for 5 min. Afterwards, 1500 µl ethyl acetate were added, vortexed for 30 s and centrifuged for 10 min at 4000 *g*. 1 ml of the upper layer (ethyl acatate) was pipetted into a 4 ml test tube and placed in a boiling water bath for 45–60 min to evaporate the ethyl acetate thoroughly. 3 ml of 1 mol/l NaCl solution was added to each tube and vortexed for 30 s. All samples were placed in a water bath at 70 °C for 5 min to redissolve all the residual hippuric acid, then vortexed for 30 s. After 15 min standing at room temperature, the absorbance of the hippuric acid was read in a 1-cm rectangular cuvette at 228 nm in a Hitachi precision spectralphotometer 100–40, with distilled deionized water as a blank.

As internal standard serum, a pool serum of normal persons was used.

Calculation of catalytic activity

1 Unit (U) of carboxypeptidase N activity is defined as the amount of enzyme required to release 1 µmol hippuric acid per min at 37 °C under standard assay conditions in 1 ml serum (i.e., U/ml = kU/l = µmol/ml · min).

$$1\ U/l = \frac{A_{228} \times 10^5 \times 3}{980 \times 0.91 \times 0.67 \times 60 \times V_n} \text{ or } \begin{matrix} A_{228} \times 167.36 \text{ for } CN_1 \\ A_{228} \times 334.72 \text{ for } CN_2 \end{matrix}$$

$$A_{228} = \frac{\text{Absorbance sample}_1 + \text{Absorbance sample}_2}{2} \; \text{l- Absorbance blank}$$

980 $m^2 \times mol^{-1}$	ε_{228} hippuric acid
0.91	fraction of extracted hippuric acid
0.67	fraction of sampled ethyl acetate
60	incubation time in min
V_n	V_{CN1} = 0.050 ml serum for CN_1
	V_{CN2} = 0.025 ml serum for CN_2
10^5	conversion to liter

Statistical calculations were performed with *Student*'s t-test. For correlation *Spearman* rank test was used.

Additionally, routine laboratory parameters were evaluated from the same serum samples used for enzymatic measurements.

Results

Since samples are stored in crushed ice before incubation, there is a time lag before the incubation mixture attains 37 °C. For this reason the course of reaction of the carboxypeptidases did not achieve linearity until after 10–20 min, using hippuryl-*L*-arginine or hippuryl-*L*-lysine as synthetic substrates. The reaction was still linear up to 120 min (fig. 1). In our routine assay we used an incubation time of 60 min, which clearly guarantees linearity.

The pH-dependence of CN_1 and CN_2 was investigated, using potassium phosphate buffer. In repeated assays CN_1 showed a small peak of activity at pH 8.4 (fig. 2). For CN_2 a broad pH optimum was found between 8.2 and 8.6. In subsequent enzymatic reactions, we also used pH 8.4 for CN_2.

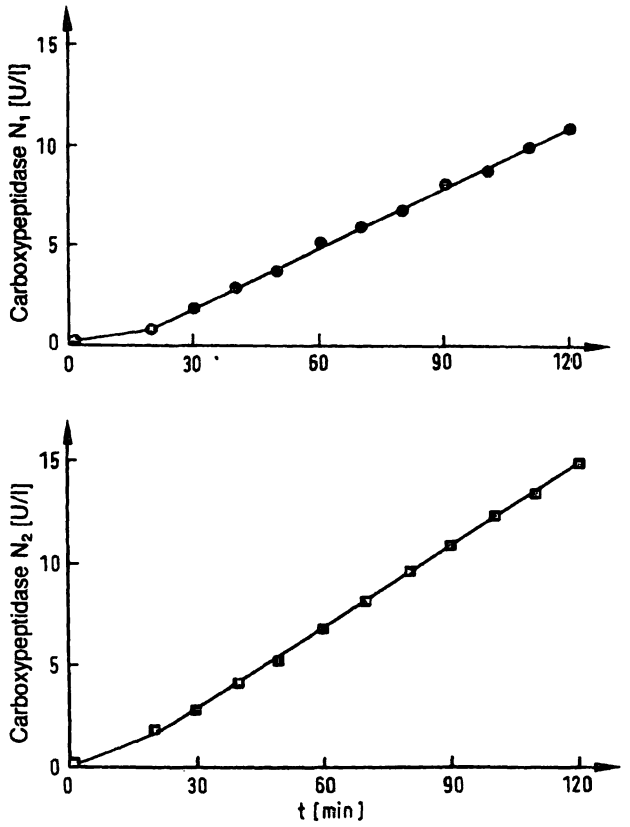

Fig. 1. Effect of incubation time on carboxypeptidase N_1 catalytic activity concentration (●——●) and on carboxypeptidase N_2 catalytic activity concentration (■——■) in serum.

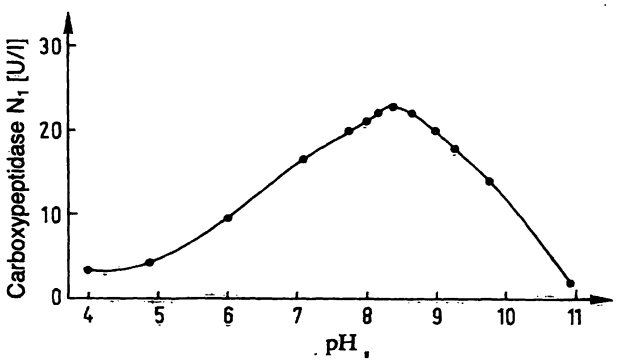

Fig. 2. Effect of pH on carboxypeptidase N_1 catalytic activity concentration in serum, measured as the rate of hydrolysis of hippuryl-*L*-arginine.

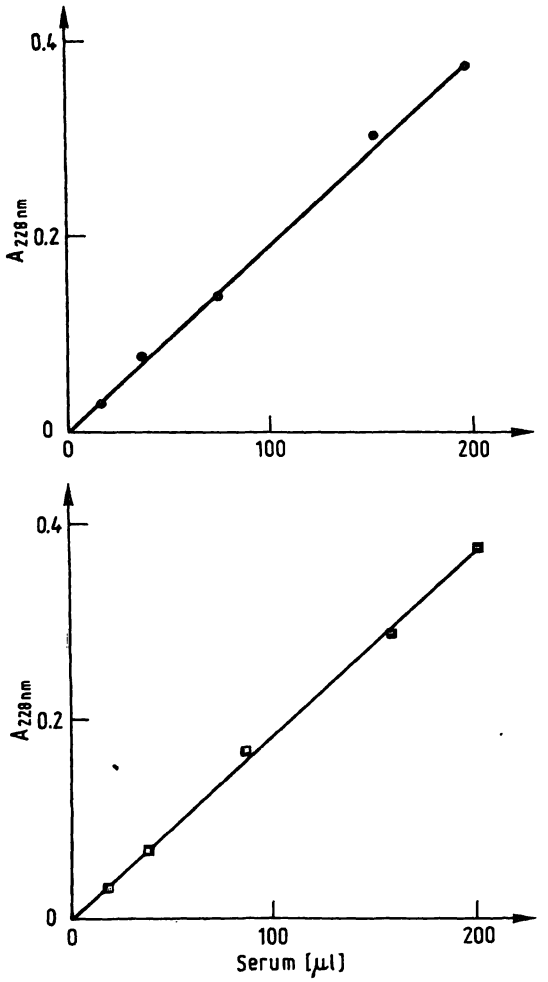

Fig. 3. Relationship between carboxypeptidase N_1 (○——○) and carboxypeptidase N_2 catalytic activity concentrations (■——■) to serum dilution.

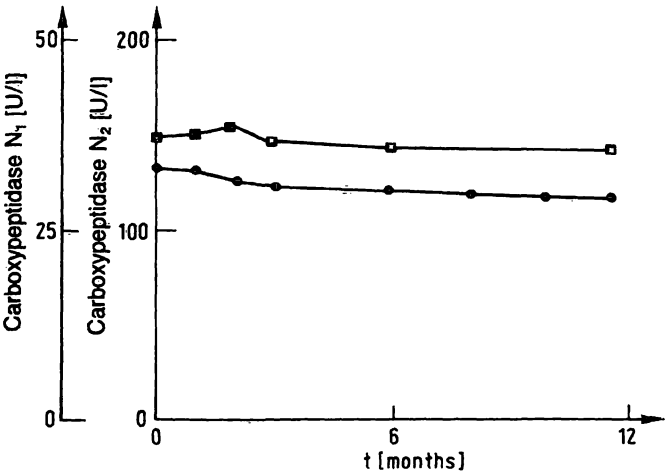

Fig. 4. Stability of carboxypeptidase N_1 (●——●) and carboxypeptidase N_2 (■——■) catalytic activity concentration in serum stored at −20 °C during 12 months.

Linearity of the assay could also be proven by serum dilution (fig. 3). CN activity was plotted as dilution factor. The linear regression analysis gave (CN_1r = 0.99, CN_2r = 0.98).

To investigate the dependence of catalytic activity on the incubation temperature, incubations were performed between +2 °C and +63 °C in a heated water bath. CN_1 showed highest activity at 40 °C, CN_2 at 41 °C (fig. 5).

The stability of CN_1 and CN_2 was tested during storage at −20 °C for up to 12 months. Only slight decreases of CN_1 (9.3%) and CN_2 (5.3%) were observed (fig. 4).

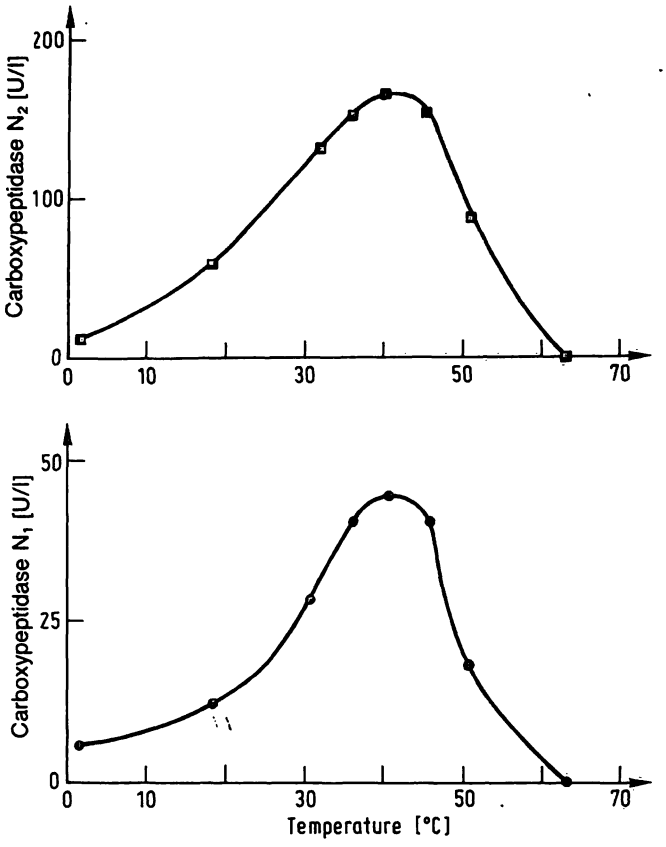

Fig. 5. Effect of incubation temperature on carboxypeptidase N_1 (○——○) and carboxypeptidase N_2 (■——■) catalytic activity concentrations in serum, measured under the assay conditions described in text.

Michaelis Menten constants (K_m) were determined by the *Linewater Burk* plot. The following K_m values were found.

CN_1: 4.59 ± 0.03 (SD) mmol/l, (n = 4),
V_{max} 19.11 ± 5.68 µmol/l · min;

CN_2: 37.29 ± 3.49 mmol/l, (n = 3),
V_{max} 147.82 ± 32.21 µmol/l · min.

Thus, the affinity of CN_1 for hippuryl-*L*-arginine is greater than the affinity of CN_2 for hippuryl-*L*-lysine.

Blocking agents were also tested (tab. 1). EDTA inhibits only CN_2 (76%) and shows no inhibition of CN_1.

CN_2 could be nearly totally blocked by dimercaprolum (97%), but CN_1 was inhibited by only 54%. Similiar results were obtained for inhibition by phenanthroline: CN_1 (64%), CN_2 (98%). Diisopropylfluorophosphate had no inhibitory effect on CN_1 or CN_2 activity, but the curves suggest a slight activation of both enzymes.

Tab. 1. Inhibitor concentrations necessary to produce 50% inhibition (I_{50}) of carboxypeptidase N_1 (CN_1) and N_2 (CN_2) measured under standard assay conditions described in the text.

	I_{50} (mmol/l)	
Blocking agent	CN_1	CN_2
Diisopropylfluorophosphate	none	none
EDTA	none	3.16
Phenanthroline	0.59	0.15
Dimercaprolum	9.70	2.41

The interference of bilirubin and haemoglobin was also tested. Initially bilirubin reduced carboxypeptidase activity slightly. Only CN_1 activity was decreased by addition of haemoglobin.

For the precision of these methods see table 2.

We measured CN_1 and CN_2 in healthy controls and in patients with various lung diseases (tab. 3). No positive correlation could be found in 47 normal subjects between cholesterol (CN_1: r = 0.04; CN_2 r = 0.08) and triglycerides (CN_1: r = 0.01; CN_2 r = 0.10). Significantly elevated activities of CN_1 were found in sera of patients with sarcoidosis ($p < 0.0001$) compared with the healthy controls and with lung cancer patients ($p < 0.002$). CN_2 was significantly increased in patients with lung cancer ($p < 0.0001$) compared with controls and with sarcoidosis patients ($p < 0.02$).

Discussion

Our interest in carboxypeptidase was prompted by our studies of the angiotensin-I-converting enzyme in lung diseases. Angiotensin-I-converting enzyme was found to be elevated in sarcoidosis (9) and liver diseases (10). Similar behaviour by other carboxypeptidases in human blood was expected.

The method described here for the determination of CN_1 and CN_2 activity in serum by spectrophotomet-

Tab. 2. Precision data of assayed serum to estimate the variability (CV %) of the method.

	Carboxypeptidase N_1 (U/l)			Carboxypeptidase N_2 (U/l)		
	Mean	SD	CV %	Mean	SD	CV %
Intraassay	30.9	± 0.6	1.8 (n = 16)	155.8	± 2.9	1.9 (n = 15)
Interassay (day to day)	30.3	± 2.1	7.1 (n = 30)	156.3	± 5.1	3.3 (n = 28)

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History of Clinical Chemistry

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Clinical Chemistry is a special discipline of medicine which, due to its close relationship both to medicine and to chemistry, is of particular interest to the historian of science.

This "History of Clinical Chemistry" is based on a modern outlook on the history of science. Since the investigation of the history of clinical chemistry is still in progress, the book is divided into eight separate contributions, written primarily by historians of science, which together provide a good coverage of the history of Clinical Chemistry in the nineteenth century.

The book is written entirely in English and will therefore appeal to an international readership. Each contribution is provided with numerous notes and references.

Contents

Tab. 3. Carboxypeptidase N_1 and N_2 catalytic concentrations in serum of patients with lung diseases compared with healthy controls. In sarcoidosis CN_1 was significantly elevated ($p < 0.002$) and CN_2 was reduced ($p < 0.02$) compared with lung cancer.

	Carboxypeptidase N_1 (U/l)			Carboxypeptidase N_2 (U/l)		
		n	p<		n	p<
Sarcoidosis	73.9 ± 32.4	40	0.0001	177.1 ± 41.8	43	0.0001
Lung tuberculosis	37.8 ± 15.7	32	0.50	138.5 ± 37.3	33	0.50
Lung cancer	54.3 ± 25.5	54	0.0001	195.3 ± 38.4	52	0.0001
Asthma	39.5 ± 12.7	18	0.50	166.9 ± 29.7	26	0.001
Pneumonia	43.2 ± 21.8	9	0.20	174.0 ± 43.6	13	0.01
Chronic bronchitis	50.1 ± 17.5	14	0.002	167.4 ± 34.4	20	0.01
Healthy controls	36.1 ± 9.9	47		144.2 ± 26.0	54	
		214			241	

ric assessment of the cleavage of synthetic substrates, is accurate and simple to perform.

Previous reports showed that human serum CN could be separated by isoelectric focusing into two subunits (12). The subunits of CN could also be distinguished by immunological methods (13). It was speculated that CN consists of two species which were apparently isoenzymes (12).

With our method, which is derived from the spectrophotometric measurement of angiotensin-I-converting enzyme (9), we also found differences in biochemical behaviour between CN_1 and CN_2. The pH curve showed a broad pH optimum when using hippuryl-*L*-lysine as substrate. The K_m-values indicate that the affinity of the enzyme activity to hippuryl-*L*-arginine is greater than to hippuryl-*L*-lysine. Only CN_2 could be inhibited by EDTA, CN_1 was not influenced by this chelating agent.

For comparison, the K_m of carboxypeptidase B, which cleaves the same substrates, is 0.21×10^{-3} mol/l for hippuryl-*L*-arginine and 7.7×10^{-3} mol/l for hippuryl-*L*-lysine (11). This shows that CN_1 removes the C terminal arginine faster than lysine. Pancreatic carboxypeptidase B acts in the same way, but the inhibition patterns show differences between carboxypeptidase B and CN.

Previous studies, using hippuryl-*L*-lysine as enzyme substrate, describe normal values of carboxypeptidase N in pregnant women and in some patients with tumours. Reduced activity was found in patients with cirrhosis of the liver (14, 15), which indicate that this enzyme may be synthesized in liver tissue. This could result in a decreased inactivation of bradykinin and other vasodilatating peptides in liver diseases. We measured CN_1 and CN_2 in patients with lung diseases and found high activities of CN_1 in sarcoidosis, and high activities of CN_2 in lung cancer compared with healthy controls. Initial investigations indicate that the elevation of CN_1 in sarcoidosis is a better parameter of clinical activity than angiotensin-I-converting enzyme. Like angiotensin-I-converting enzyme, the source of CN_1 may be the epitheloid cells. There is some evidence that CN_2 is produced by malignant tumour cells.

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